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(71) Applicant *(for all designated States except US)*: THE SEC-RETARY OF STATE FOR DEFENCE [GB/GB]; Defence Evaluation & Research Agency, Ively Road, Farnborough, Hampshire GU14 0LX (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ANDREW, David [GB/GB]; CBDE, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). LINDSAY, Christopher [GB/GB]; CBDE, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB).

(74) Agents: SKELTON, S., R. et al.; D/IPR, Formalities Section (Procurement Executive), Poplar 2, MOD Abbey Wood #19, Bristol BS34 8JH (GB).

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(54) Title: PROTECTIVE COMPOSITIONS

(57) Abstract

A composition for use in protecting against alkylating agents in particular sulphur mustard; said composition comprising a compound of the formula (I): $H_2NCH(COOR^2)CH_2CH_2CONHCH(CH_2SH)CONHCH_2COOR^1$, or a salt thereof; where R^1 and R^2 are independently selected from hydrogen or an optionally substitued hydrocarbyl group having at least three carbon atoms; provided that R^1 and R^2 are not both hydrogen, is described. Preferred compounds of the formula (I) are monoisopropylglutathione ester (MIPE) or diisopropylglutathione ester (DIPE). Compositions are suitably formulated as barrier creams and/or liquids for application by nasal spray or inhaler. Method of protecting living tissue from the effects of alkylating agent such as sulphur mustard are also described and claimed.

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Protective Compositions

5   The present invention relates to the use of certain
    glutathione ester reagents in the preparation of compositions
    for protecting personnel against toxic alkylating agents such
    as sulphur mustard (SM) and carcinogens which bind to thiol
    groups, to certain compositions obtained thereby, and to
10 methods of protection using these compounds.

    The first recorded use of sulphur mustard (HD) as a chemical
    weapon was during the First World War at Ypres in 1917, during
    which it was a major cause of military casualties. More recently
15 there have been several instances of its use by Iraq against
    Iranian and Kurdish targets.

    Exposure to HD causes debilitating skin vesication which may
    result in permanent or temporary incapacitation. Inhalation of
20 HD either in aerosol or vapour form may cause damage or
    destruction to the respiratory tract, resulting in injury or
    death. HD is able to form irreversible covalent linkages with
    many nucleophilic groups in the cell, particularly with DNA. The
    mono-functional and particularly the bi-functional intra- and
25 inter-strand cross-links formed ultimately result in the
    inhibition of mitosis and cell death. Exposure to HD also
    results in the depletion of intracellular NAD^+ levels and
    consequently glycolysis is inhibited. The enzymes of the
    pyruvate oxidase system are also sensitive to direct interaction
30 with HD.

Pretreatment of target organs such as lung tissue with nucleophiles may reduce the toxic effects of exposure to HD.

Thiol containing compounds such as cysteine esters have been found to protect rat lung slices *in vitro* (Wilde et al. Human and Experimental Toxicology (1994) 13: 743-748). The use of cysteine compounds as protective compounds is described in WO 92/04024. Glutathione ethyl ester has also been reported to protect peripheral human blood lymphocytes (PBLs) against HD (Gross et al. Proceedings of the 1989 Medical Defence Bioscience Review, US Army Medical Research and Development Command, 15-17 August 1989, Aberdeen Proving Ground, Maryland, USA 415-418 1989), and N-acetyl cysteine can protect human PBLs against the toxic effects of low concentrations of HD *in vitro* (Gross et al., Cell Biology and Toxicology, 1993, 9, 259-267).

In addition, in another context, it has been found (Kobayashi *et al.* Photochemistry and Photobiology, 63, 106-110) that pretreatment of hairless mice with monoisopropylglutathione ester (MIPE) protects against skin damage caused by exposure to UVB radiation.

Such esters have been found to act as suppressors for increased triglycerides in alcohol-damaged livers in rats (JP010063525). Furthermore, pharmaceutical compositions comprising amino acid salts of such compounds have been described in WO 93/25573.

The applicants have found that a different type of glutatione compound produces protective effects which are better than those known hitherto with such compounds.

According to the present invention, there is provided the use of
a compound of formula (I)
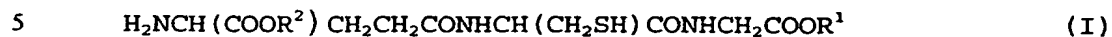
5    $H_2NCH(COOR^2)CH_2CH_2CONHCH(CH_2SH)CONHCH_2COOR^1$ (I)

or a salt thereof;
where R^1 and R^2 are independently selected from hydrogen or an
optionally substituted hydrocarbyl group having at least three
10   carbon atoms; provided that R^1 and R^2 are not both hydrogen; for
use in the preparation of a composition for protecting against
alkylating agents.

Suitable hydrocarbyl groups for R^1 and R^2 are alkyl, alkenyl,
15   alkynyl or aryl groups such as phenyl or naphthyl, having for
example from 3 to 10, preferably from 3 to 6 carbon atoms. Said
groups may include straight or branched chains.

Suitable optional substituents for the hydrocarbyl groups
20   include functional groups which do not increase the toxicity
and/or solubility of the compound of formula (I) to an
unacceptable level, or do not significantly reduce the
protective effect of the compounds. Such functional groups may
include halogen, such as fluoro, chloro, or iodo, aryl groups
25   such as phenyl, nitro, amino, mono or dialkyl amino groups
wherein the alkyl groups suitably contain from 1 to 6 carbon
atoms, oxo groups, thio groups, acyl such as acetyl, hydroxy or
alkoxy groups such as C_{1-6} alkoxy.

30   Suitable salts of the compounds of formula (I) are
pharmaceutically acceptable salts as is understood in the art.

In particular, the salts may be salts which are soluble in water such as the hydrochloride salt, to assist in formulation.

5   Preferably, R^1 and R^2 are selected from hydrogen or alkyl, and most preferably C_{3-6} alkyl.

Compounds of formula (I) are glutathione derivatives. Particularly preferred compounds of formula (I) are monoisopropylglutathione ester (MIPE) where R^1 is isopropyl and R^2
10   is hydrogen, or diisopropylglutathione ester (DIPE) where both R^1 and R^2 and isopropyl groups.

Compounds of formula (I) are known compounds or they can be derived from known compounds by conventional methods, for
15   example by esterification of glutathione.

The compositions produced in accordance with the invention suitably comprise additional reagents as is conventional in the formulation art. These include carriers, adjuvants, fillers,
20   stabilizers, surfactants, emulsifiers etc. The compositions may be in the form of liquids, solids or semi-solids depending upon the intended mode of application.

In particular, the compositions of the invention are in the form
25   of topical compositions for application to the skin. Such compositions include creams and particularly barrier creams. Compositions of this type are novel and form a further aspect of the invention.

30   Thus the compositions of the invention suitably further comprises a cream base. Examples of cream bases include oil in water emulsions, or polymethoxysiloxane or stearic acid.

Barrier creams for use on the hands may be particularly
useful, for example where work has to be done in a
contaminated area. Although protective clothing may be
5   effective in maintaining a barrier against HD, it is generally
quite cumbersome and may inhibit efficient work. By using a
barrier cream, an operator may be able to work without gloves
in such an area, at least for a limited period of time.

10   Alternatively, the compositions obtained in accordance with the
invention may be adapted for direct administration to mucosal
membranes such as lung tissue and nasal passages. Such
compositions may be in the form of nasal sprays or compositions
suitable for administration by inhalers or nebulizers such as
15   the Devilbiss nebulizer. In such cases, the compound of
formula (I) may be incorporated into structures such as vesicles
or liposomes to ensure efficient delivery to the target tissue
as are known in the art.

20   The dosage of compound of formula (I) will depend upon the
nature of the patient being treated, the seriousness of the
condition or the particular alkylating agent likely to be
encountered in accordance with usual clinical practice. In
general however, a dosage of from 5 to 60mg/kg, suitably about
25   30mg/kg would be used. The concentration of the compound within
the composition will be appropriate to deliver these dosages in
appropriate volumes.

The invention further provides a method of protecting living
30   tissue from the effects of alkylating agent, said method
comprising applying to the tissue, a compound of formula (I) as

6

described above, either before, during or after exposure to said alkylating agent.

Preferably however, the compound of formula (I) or a composition
5   containing it is applied to a subject prior to exposure to the alkylating agent in such a manner that it remains in contact with the tissue during the relevant exposure.

The A549 cell line was used, *inter alia*, as an *in vitro* model to
10 assess the efficacy of potential anti-HD agents, specifically MIPE and DIPE. This line is a human alveolar type II epithelium-like adenocarcinoma derived cell line (Asano et al., Proceedings of the National Academy of Science, USA, 1994; 91, 10089-10093, Wu et al. American Journal of Respiratory Cell and Molecular
15 Biology, 1994: 10, 437-447) and has been used in several studies on pneumocyte biochemistry (e.g Arnold et al. Immunology, 1994, 82, 126-133).

The importance of type II pneumocytes lies in their involvement
20 in alveolar repair processes. In the event of alveolar damage occuring, type II pneumocytes proliferate and replace damaged type I cells; the extracellular matrix secreted by type II cells also influences the repair process (Maniscalco et al., American Journal of Physiology, 1994, 267, L569-L577- Lung Cell Mol.
25 Physiol. 11). The use of nucleophiles to protect type II cells from toxic compounds such as HD may consequently enhance post-exposure healing processes in lung tissue.

Biochemical and morphological approaches were employed to
30 determine both the toxic effects of HD on cells and the protective properties of the above-mentioned esters including MIPE and DIPE against this chemical warfare agent. The

7

toxicology of HD in A549 cells was determined prior to
establishing an appropriate challenge dose for protection
studies.

5   High performance liquid chromatograpy (HPLC) was used to measure
the effects of MIPE on the intracellular thiol status of A549
cells.

It was found that HD rapidly reduced the viability of A549 cells
10   at concentrations greater than 40µM, with a 1 h dose of 1000µM
HD reducing A549 cell viability to 64% (GV assay) at 10 h. Lower
doses of HD increased the time to onset of toxic effects and is
an observation consistent with an agent that has numerous intra-
and extra-cellular sites of action. The results showed that
15   200µM HD was an appropriate dose for determining the efficacy of
MIPE as a protectant. At this dose of HD, cell growth within the
A549 cultures ceased, and cells became detached from the
substratum with the remainder being swollen in appearance. Cell
death was characteristic of necrosis.
20

Pretreatment of A549 cells with MIPE (followed by its removal
from the cell cultures) resulted in protection, though it
produced increased levels of viability ranging between 1.1- and
1.4-fold greater than for HD only exposed cultures.   However,
25   higher levels of protection against HD, resulting in increases
in viability of up to 2.1-fold compared to HD only exposed
cultures, were found when A549 cultures were exposed to HD in
the presence of MIPE (co-treatment). These levels of protection
are therefore higher than has been reported elsewhere for
30   protective nucleophiles such as GEE (Gross et al. 1989 supra)
and NAC (Gross et al. 1993 supra).

The morphology of cells in the protected cultures was normal. Furthermore, MIPE was also effective in protecting A549 cells if added at intervals of up to 7.5 min after the addition of HD. This suggests that HD does not react irreversibly with sensitive biological targets for several minutes, and that within this short window MIPE can effectively "rescue" cell cultures from its lethal effects. CYS levels were found to be raised to high levels by MIPE (comparable to that of non-HD treated cultures) even if MIPE was added 5 min after HD exposure. The protective effects of DIPE were found to be broadly similar to those of MIPE.

HPLC analysis of MIPE treated cultures indicated that the protection which MIPE was found to confer was not due to the raising of intracellular levels of GSH, for these levels did not rise significantly above control levels when cultures were treated with MIPE. In contrast, MIPE was found to raise intracellular CYS levels by up to 40-fold over that of control cultures.

Without being constrained by any particular mode of action, the applicants would speculate that it is more probable that the increased viability of A549 cells pretreated with compounds of formula (I) prior to HD exposure is due to these raised intracellular CYS levels, for other studies have identified compounds that raise intracellular levels of cysteine in rat lungs to levels which protect against perfluoroisobutene-induced pulmonary oedema; these include cystine dimethyl ester, cysteine methyl ester and cysteine isopropyl ester (Lailey et al.Biochemical Pharmacology, (1991) 42, S47-S54). Wilde and Upshall (supra.) also found that the methyl, isopropyl, cyclopentyl and cyclohexyl esters of cysteine proved effective

against 0.1 mM HD in protecting rat lung slices *in vitro* against HD. The protection was attributed to the raising of intracellular CYS levels; the interaction between HD and CYS resulting in lower levels of HD being available to exert toxic

5   effects on the tissue.

The increases in viability which were found to occur when co-treating cells with compounds of formula (I) and HD compared to only pretreating with compounds of formula (I) must be due to

10   additional extracellular inactivation of HD by the compound of formula (I), for the intracellular levels of CYS measured in the co-treated cells were comparable to those found in cells pretreated with a compound of formula (I). Increasing intracellular levels of protective nucleophiles such as CYS may

15   therefore not be the only effective strategy against HD.

The inability of MIPE to raise intracellular GSH levels in A549 cells, even though a mechanism exists to transport GSH substrates across the plasma membrane of type II pneumocytes, is

20   surprising. In studies elsewhere, the related compound GEE was found to raise GSH levels in PBLs. GSH protects cells against oxidant injury, and intracellular levels are known to fall in human keratinocytes after exposure to HD. Raising intracellular GSH levels may therefore be an effective strategy against HD

25   toxicity, though the present results suggest that other strategies can be more effective.

The basis of the interaction between HD and compounds of formula (I) is probably due to the presence of the thiol (-RSH) group in

30   the compounds of formula (I). Thiol groups of this type are classified as soft nucleophiles. It is the epi-sulphonium ion (ESI) of the highly reactive cyclic intermediate which is the

active principle of HD, not the β-chloroethyl structure. The ESI intermediate of HD alkylates nucleophilic residues in macromolecules, forming covalent linkages; the nucleophilic substitution reactions commonly proceed via S_N1 and S_N2 mechanisms. Sulphur nucleophiles possess the greatest affinity for the ESI and this may be an important factor in the use of thiol containing compounds to protect cells and tissues from the effects of HD. Compounds of formula (I) appear to be effective scavengers of HD in the biological system described in the present study and this is the first report of its anti-HD protectant properties.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 shows the effect of a 1h pulse of HD on A549 cell viability over time;

Figure 2 illustrates the protection of A549 cells by pre-, co- and post treatment with MIPE;

Figure 3 is a graph illustrating the protection of A549 cells by the addition of MIPE to HD exposed cultures over time;

Figure 4 shows the results of experiments to test the protective effect of compounds of formula (I) on the BEAS-2B and RPMI 2650 cell lines;

Figure 5 shows the results of experiments to test the protection of A549 cells with DIPE where A549 cells were

incubated at 37°C with either HBSS only (I), HBSS + DIPE (II),
HBSS + HD (III), and DIPE + HD (IV);

Figure 6 shows the results of experiments to test the
5   protection of A549 cells by cotreatment with DIPE where DIPE (8
mM; 100 µl) was present on the cell cultures at the time of HD
challenge (200 µM HD; 100 µl). A549 cells were incubated at 37°C
with either HBSS only (I), HBSS + DIPE (II), HBSS + HD (III),
and DIPE + HD (IV).
10

Figure 7 shows the protection of A549 cells by pretreatment and
cotreatment with DIPE where HBSS only is (I), HBSS + DIPE (II),
HBSS + HD (III) and DIPE + HD is (IV).

15   Figure 8 shows the protection of A549 cells by post-treatment
with DIPE with either HBSS only (I), HBSS + DIPE (II), HBSS + HD
(III) and DIPE + HD (IV); and

Figure 9 shows the results of an experiment to test the
20   protection of A549 cells by the addition of DIPE to HD exposed
cultures over time.

Example 1
Preparation of Esters

25

__L-Cysteine isopropyl ester hydrochloride__
Hydrogen chloride was bubbled through a stirred suspension of L-
cysteine (33 0 g, 0.27 mmol) in propan-2-ol (250 ml) and the
reaction mixture brought slowly to reflux maintaining the
30   presence of hydrogen chloride. After 5.5 h the reaction was
cooled to room temperature and stored overnight. The resultant

solid was filtered off and washed with several quantities of ether to give the pure ester (37.0 g, 68%).

V_{max} (KB,disc)3000(br), 1780(C=O).

5
^1H δ(CD$_3$OD): 5.30(1H, sep, J=7.0Hz, OC$\underline{H}$), 4.50(1H, m, C$\underline{H}$N), 3.30(2H,m, C$\underline{H}_2$S).

^{13}Cδ(CD$_3$OD): 167.0(C=O), 72.5(O$\underline{C}$H), 55.8($\underline{C}$HN), 25.2($\underline{C}$H$_2$S), 21.8($\underline{C}$H3).

<u>t-Boc-cystine</u>

10
To a stirred solution of cystine (15.4 g, 64.0mmol) in *tert*-butanol was added NaOH (5.13 g, 128.0mmol) in water (55 ml) followed by di-*tert*-butyl dicarbonate (28 g, 128mmol). After 5 hours, the mixture was extracted with ether (3 x 75 ml), the aqueous phase was adjusted to pH 2 and the resultant solid

15
filtered off and dried to give the pure protected amino acid (15.0 g, 50.5%).

^1Hδ(CD$_3$OD): 4.40(2H,m,C$\underline{H}$N), 3.23(2H,dd,J=4.49 and 10.7Hz,C$\underline{H}_2$S), 2.95 (2H,dd,J=8.70 and 10.7Hz,C$\underline{H}_2$S), 1.44(6H,s, C$\underline{H}_3$).

^{13}Cδ(CD$_3$OD): 174.1(O$\underline{C}$=O), 157.8(N$\underline{C}$=O), 80.7($\underline{C}$(CH$_3$)$_3$), 54.7($\underline{C}$H),

20
28.7($\underline{C}$H$_3$).

<u>N-α-t-Boc-L-glutamic acid α-isopropyl-γ-benzyl ester</u>

A solution of N-α-t-Boc-L-glutamic acid-γ-benzyl ester (10.0 g, 29.6 mmol), DBU (4.5 g, 32 mmol), 2-bromopropane (4.0g, 32mmol)

25
and a catalytic amount of tetrabutylammonium bromide (100 mg) in dry THF (150 ml) were heated under reflux. After 9 hours the solvent was removed under reduced pressure and the residue dissolved in CH$_2$Cl$_2$ (150 ml). The organic layer was washed with water (100 ml), HCl (1M, 100 ml), NaHCO$_3$ (10% solution, 100 ml),

30
water (100 ml) in turn, dried over anhydrous MgSO$_4$ and concentrated at reduced pressure to give pure product (11.0 g, 98%).

^{1}Hδ(CDCl$_3$), 7.30(5H m Ar), 5.1 (2H,s ArCH$_2$), 5.05 (1H,br NH), 5.05
(5H, sept,J = 5Hz,CHO), 4.25 (1H,m(br),CHNH), 2.40
(2H,m(complex),CH$_2$CH$_2$C=O), 2.15-1.95 (1H each,m(br) CH$_2$CH$_2$C=O),
1.41 (9H,s,(CH$_3$)$_3$),1.23-1.22 (3H each,d,J=5 Hz (CH$_3$)$_2$).

N-α-t-Boc-L-glutamic acid α-isopropyl ester

The t-boc protected benzyl ester (7.0 g, 18.5 mmol) was
dissolved in THF (150 ml), a catalytic amount of 10% palladium
on charcoal added and the mixture hydrogenated until the uptake
of hydrogen ceased. The suspension was filtered and the
filtrate concentrated at reduced pressure to give pure product.
δ^1H (CDCl$_3$): 5.20 (1H,(br)NH), 5.02 (1H, sept, CHO), 4.20 (1H,
br, CHN) 2.50, (2H, m, CH$_2$C=O), 2.00 (2H, br, CH$_2$CH$_2$), 1.41 (9H,
s, (CH$_3$)$_3$), 1.22-1.24 (3H each, 2d's (CH$_3$)$_2$).
^{13}Cδ (CDCl$_3$): 177.5, 177.1 (C=O, CO$_2$H and CO$_2$) 155.5 (NC=O), 80.1
(OC(CH$_3$)$_3$), 69.4 (CHO), 67.9 (CHN), 30.1 (CH$_2$CO=O), 25.6 (CH$_2$CH),
30.1, 28.4 ((CH$_3$)$_2$), 28.3 ((CH$_3$)$_3$).

t-Boc-Cystine-Gly-isopropyl ester

To a stirred suspension of t-boc-L-cystine (6.4g, 138 mmol) in
dry THF (100 ml) at -10°C N methylmorpholine (3.0 ml, 27.6 mmol)
and isobutylchloroformate (3.6 ml, 27.6 mmol) were added. The
mixture was stirred at -10°C for 12 min then glycine isopropyl
ester hydrochloride (4.3 g, 27.6 mmol) and N-methylmorpholine
(3.0 ml, 27.6 mmol) were added and the mixture allowed to warm
to room temperature. After stirring for a further 18 h the
solvent was removed at reduced pressure and the residue
dissolved in a mixture of ethyl acetate (400 ml) and a 10%
NaHCO$_3$ (100 ml). The organic layer was washed with water (100
ml), HCl (1 M, 100 ml) and water (100 ml) in turn, dried over
anhydrous MgSO$_4$ and concentrated at reduced pressure.

Recrystallisation of the residue from petrol ether / ethyl
acetate gave the pure peptide (6.5 g, 69%).

^{1}Hδ (CDCl$_3$): 8.10 (2H,br,CH$_2$NH), 5.50 (2H,d,J=9.7Hz,CHNH) 5.00
(2H,sep,J=6.2Hz,OCH), 4.90 (2H,t(br),NHCH), 4.10 (2H,dd,J=6.6
and 17.3Hz,NCH$_2$), 3.80 (2H,dd,J=5.5 and 17.4Hz,NCH$_2$), 3.07
(2H,dd,J=4.0 and 14.7Hz,SCH$_2$), 2.90 (2H,dd,J=1.8 and
14.7Hz,SCH$_2$), 1.40 (18H,s,C(CH$_3$)$_3$), 1.25 and 1.24 (12H,d,C(CH$_3$)$_2$).

Cystine-gly-isopropyl ester

Trifluoroacetic acid (20 ml) and triethylsilane (8 ml) were
added to a stirred solution of the peptide (6.5g, 10mmol) in dry
CH$_2$CI$_2$ (40 ml). After storing at room temperature 15 h the
mixture was concentrated at reduced pressure, triturated with
ether and the resultant solid washed with several quantities of
ether to give the pure unprotected peptide TFA salt (7.0 g,
98%).

^{1}Hδ(CD$_3$OD): 5.05(2H,sep,J=6.5Hz,(CH$_3$)$_2$CH), 4.30(2H,m,CHN),
4.0(1H,dd,J=17.5Hz,NCH$_2$), 3.1,3.45(2H each, SCH$_2$),
1.25(12H,d,J=6.5Hz,C(CH$_3$)$_2$).

Boc-glu-cystine-gly-diisopropyl ester

To a stirred solution of t-boc glutamic acid α isopropyl ester
(2.6 g, 9.78 mmol) in dry THF (50 ml) at -5°C under argon were
added N-methymorpholine (1.1 ml, 9.78 mmol) and
isobutylchloroformate (1.3 ml, 9.78 mmol). After 15 min a
solution of cystine-gly isopropyl ester TFA salt (2.1 g, 4.8
mmol) in dry THF (10 ml) was added and the suspension brought to
room temperature. After stirring for a further 17 h the mixture
was concentrated at reduced pressure and the residue dissolved
in a mixture of ethyl acetate (300 ml) and 10% NaHCO$_3$ (100 ml).
The organic extract was washed with H$_2$O, HCI (10% solution), and

H₂O in turn then dried over MgSO₄ and concentrated at reduced pressure. Purification by column chromatography (silica gel,CH₂Cl₂-MeOH9.8:0.2) gave the pure tripeptide (2.4 g, 51%).

5   ¹Hδ(CDC13): 8.44(2H,br,NHCH₂C=O), 6.85(2H,d(br)NHC=O), 5.47, (2H,t(br),OC=OCHN), 5.30 (2H,d(br)NHCHCH₂S), 5.05 (4H,2septs,J=5Hz,CHO), 4.31(2H,t(br),NHCHCH₂S), 4.05(2H,dd,J=18.1, 6.0 HZ(CH₂N), 3.88(2H,dd,J=17.9, 4.9Hz,CH₂N), 3.05(2H,dd,J=15.5,CH₂S), 2.90(2H,dd,J=15,11Hz CH₂S),

10   2.35(4H,t,J=7Hz,CH₂C=O), 2.20-1.95(2H each,br,CH₂CH₂C=O), 1.40(18H,S,(CH₃)₃), 1.23-1.21(12H each,2d's,J=5.5Hz,(CH₃)₂).

Glu-cystine-gly-diispropyl ester (oxidised glutathione diisopropyl ester)

15   Trifluoroacetic acid (5.0 ml) and triethylsilane (2.5 ml) were added to a stirred solution of the protected peptide (1.4 g, 1.43 mmol) in dry THF (10 ml). After 17 h the mixture was concentrated at reduced pressure and the residue triturated several times with pet ether to give the pure deprotected

20   peptide TFA salt (1.4 g, 97%).

     ¹Hδ(CDCl₃): 7.38(1H,s(br),NHCH₂), 6.92(1H,d,J=8.1Hz,NHCH), 5.01 (2H, two seps,J=6.0Hz,CHO), 4.65 (1H,ddd,J=4.0,5.6,8.3 Hz,CHCH₂S), 4.10(1H, dd, J=6.0, 15.9, CH₂N), 3.80, (1H,dd,

25   J=4.4,18.1,CH₂N), 3.40(1H,dd,J=4.4,9.1,CHNH₂), 3.20(1H,dd,J=4.6,13.9,CH₂S), 2.67(1H,dd,J=5.7,13.9,CH₂S), 2.35(2H,m,J=obs,CH₂CHN), 2.10(2H,m,J=obs,CH₂C=O), 1.30(12H,d,J=6.0Hz,CH₃).

30   ¹³Cδ (CDCl₃): 175.0,172.7,170.1,169.0(4xC=O's), 69.4,68.8(2xCHO's), 54.6(CHNH₂); 53.7(CHCH₂S), 41.6(CH₂N), 33.7(CH₂CHN), 29.6(CH₂C=O), 26.6(CH₂S), 21.8(CH₃).

Glutathione diisopropyl ester (reduced glutathione diisopropyl
ester)

The oxidised peptide TFA salt (1.4 g, 1.39 mmol) was dissolved
in water (10 ml) and the pH adjusted to 7 by the addition of
10%NaHCO, the solution was stirred at room temperature and
dithiothreitol (0.27 g, 1.75 mmol) was added. After 17 h the
mixture was analysed by reverse phase HPLC. The column used was
a Vydac protein and peptide C18, the mobile phase was solvent A
(95% water, 5% TFA) and solvent B (95% acetonitrile, 5% TFA).
The solvent programme comprised 0-2 min 5% solvent B, 2-25 min
linear gradient of 5-50% solvent B, 25-29 min linear gradient of
40-90% solvent B, 29-32 min 90% solvent B, 32-35 min linear
gradient of 90-5% and 35-40 min 5% solvent B at a rate of
1ml/min. The eluate was monitored using a uv dector at
wavelengths 224 and 254 nm. The product had a retention time of
17.5 min and there was no evidence of any starting material.
The reation mixture was acidified to pH 2 by the addition of 10%
HCl, extracted with ether (x3) and the extracts discarded. The
aqueous phase was adjusted to pH 7.5 and extracted with ether
(x8). The combined extracts were dried over MgSO, and
concentrated at reduced pressure to give the title compound
(0.66 g, 48%).

Glutathione diisopropyl ester

To a stirred solution of glutathione (25.0 g, 81.4 mmol) in
sodium dried propan-2-ol (250 ml) under argon at room
temperature concentrated H_2SO_4 (12.5 ml) was added dropwise.
After storing at room temperature 3 days the mixture was
analysed by HPLC, conditions as above. The chromatogramme
showed the presence of 4 products identified as both monoesters,
the diester and a cyclised product. The mixture was

concentrated at reduced pressure, the pH adjusted to 7.5 by the
addition of 10% NaHCO$_3$ and extracted with ether (x3). The
extracts were combined, dried and concentrated at reduced
pressure to give the ester slightly contaminated with oxidised

5   material. The impure ester was dissolved in a minimum amount of
water, the pH adjusted to 7 by the addition of 10% HCl and
excess dithiothreitol added. After 17 analysis by HPLC,
conditions as above, showed the reaction to be complete. The
mixture 7 was acidified to pH 2 extracted with ether (x3) and

10 the extracts discarded. The aqueous phase was then adjusted to
pH 7-8, extracted with ether (x3) and the combined extracts
dried over MgSO$_4$. Concentration at reduced pressure gave the
title compound (5.1g, 15.8%).

15 <u>Glutathione mono-isopropyl H$_2$SO$_4$ ester</u>
To a stirred solution of glutathione (10.0 g, 32.6 mmol) in
propan-2-ol(sodium dried, 100 ml) under argon at room
temperature, concentrated H$_2$SO$_4$ (2.74 ml) was added dropwise.
After stirring at room temperature 18 hrs the mixture was

20 diluted with diethyl ether (sodium-benzophenone dried, 100 ml)
stored at -4°C to crystallise glutathione mono-isopropyl H$_2$SO$_4$
ester. After 2 days the mixture was filtered and the crystals
washed with diethyl ether (3 x 100 ml) to give the title
compound (14 g, 93%).

25

^{1}Hδ(CD$_3$OD) : 5.05(1H,sep,J=6.0Hz,C$\underline{H}$O),
4.56(1H,dd,J=5.5,7.0Hz,C$\underline{H}$CH$_2$S), 4.07(1H,m,C$\underline{H}$NH$_2$),
3.90(2H,dd,J=17.7Hz,C$\underline{H}_2$NH), 2.87(1H,dd,J=5.5,14.0Hz,C$\underline{H}_2$S),
2.84(1H,dd,J=7.3,14.0Hz,C$\underline{H}_2$S), 2.60(2H,t,J=7.4Hz,C$\underline{H}_2$C=O),

30 2.20(2H,m,C$\underline{H}_2$CH$_2$C=O), 1.25(6H,d,J=6.0Hz,C$\underline{H}_3$).

¹³Cδ(CD₃OD) 174.5, 172.8, 171.4, 170.6(4x$\underline{C}$=O'S), 70.3($\underline{C}$HO),
57.1($\underline{C}$HCH₂S), 53.6($\underline{C}$HNH₂), 42.4($\underline{C}$H₂N), 32.3($\underline{C}$H₂CHN), 27.0($\underline{C}$H₂C=O),
25.2($\underline{C}$H₂S), 22.0($\underline{C}$H₃).

5

Example 2

Cell Cultures

The A549 cell line (no. 86012804) was obtained from the European
Collection of Animal Cell Cultures, Centre for Applied
Microbiology and Research, Porton Down, Salisbury, UK.

10

The cell line growth medium consisted of DMEM containing FCS
(10%, v/v), penicillin G (100 IU/ml), streptomycin (100 mg/ml),
and glutamine (2 mM); this is referred to subsequently as DMEM
medium. Cells were grown in a humidified atmosphere of 5%

15 CO₂/95% air at 37°C. They were harvested using trypsin/EDTA in
log growth phase for use in toxicology studies.

The A549 cells were seeded into 96-well plates at a density of 5
x 10⁴ cells/well in DMEM medium. The cells were allowed to

20 adhere over a period of 24 hours (h) prior to treatment with HD.
Exposure to HD and to MIPE was carried out in the 96-well
plates. HD dilutions in HBSS were prepared immediately
beforehand from a stock solution of 40 mM HD in propan-2-ol.

25   Example 3

Determination of the effect of pulses of HD on A549 cells

The onset of HD-induced toxicity in A549 cell cultures was
determined by measuring the viability of cultures over time
using the genetian violet (GV) assay. Cellular density in A549

30 cultures was determined using GV (Gilles et al., Analytical
Biochemistry, 1986; 159: 109-113) and was used as a cell
viability assay as described by Griffiths et al Toxicology,

1994: 90: 11-27; the contents of which are incorporated herein by reference.

To determine the effects of exposure to HD, the DMEM medium
5   overlying the cells was removed and solutions of HBSS (100µl) containing 0 to 1000µM HD were added to each well. The cultures were exposed to the HD solutions for 1 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. The HD solutions were removed and 100µl of DMEM medium added. The cultures were further incubated
10   for periods of up to 72 h under the conditions described above. At the end of each time point, the GV dye assay was carried out to determine the viability of the cultures. Viability under experimental conditions was related to that of control cells exposed to HBSS only (the reference 100% viable cell control).
15   The data is presented in Figure 1 as mean values +/- SD (n=10). In this Figure, results using HBSS solutions containing 8(▲), 40(●), 200(▼), 400(♦) and 1000(■)µM HD are illustrated.

The results show that at 10 h, the interpolated viability of
20   cultures exposed to 1000, 400, 200, 40 and 8 mM HD was 64%, 76%, 77%, 93% and 91% respectively; at 30 h it decreased further to 24%, 37%, 43%, 71% and 84% and at 50 h had fallen to 14%, 24%, 27%, 74% and 80%. The onset of toxic effects was therefore a function of HD dose, with a rapid onset at high concentrations
25   of HD and a slower onset at low concentrations of applied HD.

Example 4

The protection of A549 cells by MIPE

The ability of MIPE to protect A549 cells exposed to an LC_{50} dose
of HD was determined by removing DMEM medium from cell cultures,
followed by adding MIPE in HBSS to the wells of each 96 well
plate. HBSS +/- HD was added to the wells after, during and
before treatment with HBSS +/- MIPE. A total of 48 wells/plate
were used. The cultures were incubated with these solutions for
1 h each at 37°C (5% CO_2/95% air), after which the solutions were
replaced with 100μl of DMEM medium and further incubated at 37°C
for a total of 24 and 48 h, at which times viability was
determined by both GV assay described above, and also the NR
cell viability assay.

The NR assay was used in the present study as an index of
viability and is described in detail by Griffiths et al.
Toxicology, 1994, 90: 11-27, the content of which is
incorporated herein by reference. M199 medium was used when
employing the neutral red (NR) dye system to avoid possible
interference from the phenol red present in DMEM. The dye
accumulates in the lysosomes of viable cells.

Four sets of experiments were conducted in which the treatments
were added in the following combinations: (a) the MIPE was put
on the cultures for 1 h then removed and the cell layer washed
once with 100μl HBSS, prior to the addition of HD for a further
1 h; (b) the MIPE was present on the cell cultures at the time
of HD challenge; (c) the HD was added to the cultures, followed
by the immediate addition of MIPE for a further 1 h; (d) the

cultures were incubated with HD for 1 h, followed by the
addition of MIPE for a further 1 h. The effects of these
treatments was compared to cultures treated with HBSS, MIPE and
HD alone (12 wells per treatment). The final concentration per

5   well was 8 mM for MIPE and 100µM for HD (total volume; 200µl).
NR and GV stained cultures were photographed using an inverted
microscope equipped with phase contrast optics at a
magnification of x200.

10 In order to probe the efficacy of immediate post-treatment of
HD-exposed cells with MIPE, a further set of experiments was
carried out to determine if adding MIPE at various times after
the application of HD to the cultures would reduce the toxicity
of HD. In this work, 100 µl HD (200µM) was added to the wells of

15 a 96-well plate, and at intervals of up to 15 min, 100 µl MIPE
(16 mM) in HBSS was then added to give final concentrations of
100 µM HD and 8 mM MIPE. The effect of this treatment was
compared to cultures treated with HBSS, MIPE and HD alone. The
cultures were treated with these solutions for a total of 1 h at

20 37°C (5% CO_2/95% air), after which the solutions were removed.
The cell layer was washed with 100 µl HBSS and 100 µl of DMEM
medium added to the wells and further incubated as above.
Viability was subsequently determined at 48 h by the GV assay.

25 For both these experiments illustrative data are presented in
Figure 2 as mean values +/- SD (n=12). A Student's two-tailed t-
test (unpaired) was used to determine if any increase in
protection obtained by treating cultures with MIPE before during
or after HD exposure was significant compared to HD only treated

30 cultures.

22

Using the NR assay, it was found that cultures of A549 cells treated with MIPE (final concentration 8 mM) during exposure to HD (final concentration 100μM) were 1.8- and 1.9-fold more viable than HD treated cultures at 24 h and 48 h respectively

5 (Figure 2a(1)). This was similar to the results obtained with the GV assay, where the viability of the cultures treated with MIPE prior to exposure to HD was 1.6- and 2.1-fold greater at 24 h and 48 h respectively compared to cultures treated with HD only (Figure 2b(1)). All these increases in viability were found

10 to be extremely significant ($p < 0.0001$).

When cultures were treated with MIPE for 1 h which was then washed off, with HD being subsequently added, lower levels of protection were found with cultures being 1.3-fold ($p < 0.001$) and

15 1.4-fold ($p < 0.0001$) more viable (NR assay) at 24 h and 48 h respectively (Figure 2a(2) than HD only treated ones. These lower levels of protection are reflected in the results for the GV assays in which the viability of cultures was 1.1-fold (difference not significant) and 1.2-fold ($p < 0.05$) greater at 24

20 h and 48 h respectively (Figure 2b(2)).

A549 cultures were also treated with MIPE immediately after addition of HD. High levels of viability were measured using the NR assay with culture viability being 1.4- and 2.4-fold greater

25 at 24 h and 48 h respectively compared to HD-only treated cultures (Figure 2a(3)). This was similar to the results obtained with the GV assay, where the viability of the cultures treated with MIPE after prior exposure to HD was 1.5- and 2.0-fold greater at 24 h and 48 h respectively compared to cultures

30 treated with HD only (Figure 2b(3)). All these increases in viability were found to be extremely significant ($p < 0.0001$).

Addition of MIPE after 1 h HD exposure of the cells to HD
resulted in small increases in viability of 1.3-fold using the
NR and GV assays at 24 h (p<0.005). No increase in viability was
measured with either assay at 48 h compared to HD only treated
5   cultures (results not shown).

Photomicrography in particular of the GV stained cultures
confirmed the results of the biochemical analyses. Cell cultures
treated with HD and no MIPE were substantially depleted of
10   cells, and contained many cells of abnormal, swollen appearance
which did not appear to be dividing. The cells in A549 cultures
protected with MIPE at the time of exposure to HD exposure,
whilst being of lower density than control cultures had a normal
morphology, and were comprised of actively dividing cells. The
15   control cultures treated with HBSS + MIPE (8µM) had the same
morphology as control cultures treated with HBSS only.

                           Example 5
20   <u>Determination of the intracellular non-protein thiol status of
A549 cells by HPLC following treatment by MIPE and HD</u>

The effect of MIPE treatment upon the intracellular reduced
glutathione (GSH) and cysteine (CYS) status of A549 cultures
25   grown in 75 cm^2 culture flasks was determined by HPLC according
to a method described by Lailey et al.Biochemical Pharmacology,
(1991) 42, S47-S54. In these studies, the DMEM medium from A549
cell cultures was removed and the cell layers washed by washing
them with 3 x 10 ml HBSS prior to any treatments being carried
30   out.

The biochemical basis of protection of A549 cultures by MIPE
against HD was measured by treating cultures of A549 cells for 1
h at $37^{\circ}C$ (5% CO_2/95% air) with: (i) 10 ml HBSS; (ii) 10 ml MIPE
(8 mM); (iii) 5 ml MIPE (16 mM) immediately followed by the

5   addition of 5 ml HD (200µM); (iv) 10 ml HD (100 µM). The effect
of delayed application of MIPE following HD exposure on the
intracellular thiol status of the cultures was determined by
treating cultures with (v) 5 ml HD (200µM) for 5 min, followed
by addition of 5 ml MIPE (16 mM); these cultures were incubated

10   for a total of 1 h as above. Prior to harvesting, the cell
layers were washed as described above. The cell layers were
scraped off the substratum in the presence of 900µl of a buffer
(50 mM N-ethylmorpholine/HCl; pH 8.0) containing 0.5 mg/ml
monobromobimane. The cell suspension was homogenised and

15   incubated in the dark for 2 h at $20^{\circ}C$. 100µl of an aqueous
solution of sulphosalycylic acid (6%, w/v) was added to the
suspension and centrifuged at 13,000 rpm for 30 min at $4^{\circ}C$. The
supernatants were decanted and filtered through 0.2µm filters
into HPLC vials. The method was based on the derivatization of

20   thiols with monobromobimane to form fluorescent groups. The
·supernatant samples which contained the adducts were injected
via a Perkin Elmer (PE) ISS200 autosampler onto a 0.46 cm x 15
cm, 3µm ODS C_{18} column equipped with a Corasil guard column. The
ODS column was coupled to a PE LC250 binary high pressure liquid

25   chromatography (HPLC) pump working at 3000 psi. Elution was
stepped using 91% of a mixture A (0.25% glacial acetic acid,
99.75% HPLC water; adjusted to pH 3.7 with NaOH (0.1 M)) and 9%
acetonitrile for 7 min followed by 60% of mixture A, and 40%
acetonitrile for 10 min. A 10 min re-equilibration with the

30   initial eluent was allowed before injection of subsequent
samples. Fluorescent derivatives were detected on a PE LS-4
spectrofluorimeter coupled to an Amstrad PC3386 operating PE

Nelson software package 2100. The GSH and CYS concentrations of
the samples were measured against standard curves of up to 100µg
for each thiol compound. The concentrations of GSH and CYS in
the samples from both studies were normalised against total

5   protein (determined by the Pierce Micro BCA Protein Assay
Reagent Kit, product no. 23235). For each treatment, the results
are presented as mean values +/- SD (n=5). Statistical
significance was determined by Welch's approximate two-tailed t-
test.

10

The results are shown in Table 1.

Table 1

                            CYS   GSH

15

                                    (µg/mg protein)
Treatment:
    i) HBSS   0.447 +/- 0.092   -   32.6 +/- 2.4   -
    ii) MIPE   16.0 +/- 1.9   p<0.0001   34.1 +/- 2.9 NS
20 iii) MIPE + HD 17.9 +/- 1.2   p<0.0001   36.7 +/- 6.7 NS
    iv) HD   0.298 +/- 0.055   p<0.05   27.5 +/- 3.5 p<0.05
    v) HD + MIPE   17.9 +/- 1.8   p<0.0001   33.4 +/- 2.0 NS

For each treatment, the results are presented as mean values +/-
25   SD (n=5); NS = not significant.

As can be seen from these results, pretreatment of A549 cells
for 1 h with MIPE was found to raise intracellular CYS levels by
35.8-fold (p<0.0001) (Table 1). In cultures treated with MIPE in
30   combination with HD, intracellular levels of CYS remained high
(40-fold greater than in control cultures; p<0.0001). Treatment
of A549 cultures with HD reduced CYS levels by a factor of 0.67-

fold ($p<0.05$). Intracellular CYS levels were raised by MIPE (36.8-fold greater than control levels; $p<0.0001$) after adding it to cells 5 min after they had been exposed to HD.

5   MIPE pretreatment of A549 cells for 1 h did not significantly raise intracellular concentrations of GSH. Similarly, MIPE pretreatment of cells prior to HD exposure and co-treatment of cultures simultaneously with MIPE and HD for 1 h did not significantly increase intracellular glutathione levels compared
10   to control cultures. Exposure to HD only decreased intracellular GSH levels by 0.84-fold ($p<0.05$) compared to control cultures.

Example 6

Maintainance of the viability of A549 cell cultures by MIPE after periods of exposure to HD

15

In order to probe how effective MIPE was at maintaining the viability of cultures exposed to HD, A549 cultures were treated with 100μl HD (200μM). The same volume of MIPE (16mM) in HBSS (IV) or with HBSS only (III) was subsequently added to the
20   cultures at intervals of up to 15 min. Control cultures were treated with 200 μl HBSS only (I) or 200μL MIPE (8mM) in HBSS(II). After incubation for 1h at 37°C (5% CO_2/95% air), the solutions were removed and the cultures were further incubated in DMEM medium and viability determined at 48h using the GV
25   assay. The results for up to the 12.5 min interval are shown in Figure 3. (Data points represent mean values, +/- standard deviation, n=12).

The viability of the cultures exposed to HD for 0.5, 2.5 and 5
30   min prior to treament with MIPE was 2.1-, 1.9- and 2.0-fold greater respectively than the HD only treated cell cultures ($p<0.0001$). After 5 min, cell viability declined, so that at 7.5

min it was 1.5-fold greater (p<0.0001) than that of unprotected cultures. After 7.5 min, cell viability in MIPE treated cultures fell to levels comparable to that of HD only exposed cultures.

5   Example 7

                   Protection of Alternative Cell lines

Using similar methodology to that described in Example 4 above, the protective effects of MIPE and DIPE (di-isopropyl ester of glutathione) was tested on two different cell lines.

10   These were the BEAS-2B, a human bronchial epithelial cell line (virally transformed) and RPMI 2650, a human nasal epithelial cell line (probably derived from a tumour).

Cells were cultured as described in Example 2 above and cell

15   viability was measured using neutral red (NR) and gentian violet (GV) assays.

Cells were exposed (in 96-well plates) to a solution of protectant compound (MIPE or DIPE) for 1 hour, a solution

20   containing sulphur mustard was then added. Protectant and sulphur mustard were removed after a further hour, cells were cultured for 48 hours before viability assays were performed. By way of comparison, cells were treated either with mustard alone, or with protectant compound alone. Control cells were

25   exposed to HBSS.

The results are shown in Figure 4 hereinafter. In these tables, the first column represents the viability of cells treated with HD alone, the second column represents cells

30   treated with both HD and protectant, the third column represents cells treated with protectant alone and the fourth column is the control group.

In Figure 4A, BEAS-2B cells were pretreated with MIPE (100
μg/ml) for 1 hour, then exposed to 50 μM sulphur mustard for 1
hour. Viability was measured after 48 hours by gentian violet
5   assay.

In Figure 4B, RPMI 2650 cells were pretreated with DIPE (100
μg/ml) for 1 hour, then exposed to 100 μM sulphur mustard for 1
hour. Viability was measured after 48 hours by neutral red
10   assay.

In Figure 4C, BEAS-2B cells were pretreated with DIPE (100
μg/ml) for 1 hour, then exposed to 30 μM sulphur mustard for 1
hour. Viability was measured after 48 hours by gentian violet
15   assay.

These results clearly show that the viability of all cells
treated with either protectant is improved as compared with
untreated cells.
20

Example 8
The Protection of A549 cells by DIPE
The methodology of Example 4 was repeated using DIPE in place
of MIPE. In this case, five sets of experiments were therefore
25   conducted in which the treatments were added in the following
combinations: (a) the DIPE (4 mM; 200 ml) was put on the
cultures for 1 h then removed and the cell layer washed once
with 100 ml HBSS, prior to the addition of HD (100 mM; 200 ml)
for a further 1 h; (b) the DIPE (8 mM; 100 ml) was present on
30   the cell cultures at the time of HD challenge (200 mM HD; 100
ml);(c) DIPE (8 mM; 100 ml) was put on the cultures for 1 h
after which HD (200 mM; 100 ml) was added for another 1 h (in

the presence of DIPE); (d) HD (200 mM; 100 ml) was added to the cultures, followed by the immediate addition of DIPE (8 mM; 100 ml) for a further 1 h; (e) the cultures were incubated with HD (100 mM; 200 ml) for 1 h, followed by the addition of DIPE (4

5   mM; 200 ml) for a further 1 h. The effects of these treatments was compared to cultures treated with HBSS, DIPE and HD alone (12 wells per treatment). The final concentration per well was 4 mM for DIPE and 100 mM for HD (total volume; 200 ml). GV stained cultures were photographed using an inverted microscope equipped

10   with phase contrast optics (magnification; x200).

Using the NR and GV viability assays as described above, and the thiazolyl blue (MTT) assay as described by Griffiths et al., Toxicology, 1994, 90: 11-27, it was found that cultures of A549

15   cells pretreated with DIPE for 1 h (and subsequently removed) prior to treatment with HD were 1.2-, 1.4- and 1.3-fold more viable respectively than HD treated cultures (p<0.0001) at 48 h (Figure 5).

20   When cultures treated with DIPE were immediately exposed to HD, the viability of the cultures was 1.6-, 1.5- and 2.0-fold greater at 48 h as determined by the MTT, NR and GV assays respectively, compared to cultures treated with HD only (p<0.0001; Figure 6).

25

A549 cultures were also treated with DIPE for 1 h prior to the addition of HD for a further 1 h (without the DIPE being removed). High levels of viability were measured using the MTT, NR and GV assays; protected cultures were 2.6-, 2.9- and 2.3-

30   fold more viable respectively at 48 h than HD only treated ones p<0.0001; Figure 7).

High levels of viability in A549 cell cultures were also
measured when they were treated with DIPE immediately after the
addition of HD to the cultures. The viability of these cultures
as determined by MTT, NR and GV assays was 1.6-, 1.6- and 2.1-
5   fold greater respectively than HD only treated cultures
($p<0.0001$; Figure 8). The addition of DIPE 1 h after exposure of
the cells to HD resulted in no increase in viability being
measured by the MTT assay, and increases of only 1.1- and 1.3-
fold with the NR and GV assays respectively, compared to HD only
10   treated cultures (results not shown).

Photomicrography of GV stained cultures confirmed the results of
the biochemical analyses. A control culture of A549 cells at 48
h was photomicrographed as was cell cultures treated with HD and
15   no DIPE. This culture was substantially depleted of cells, and
contained many cells of abnormal, swollen appearance which did
not appear to be dividing. The cells in A549 cultures protected
with DIPE at the time of exposure to HD, whilst being of lower
density than control cultures had a normal morphology, and were
20   comprised of actively dividing cells. The control cultures
treated with HBSS + DIPE (8 mM) had the same morphology as
control cultures treated with HBSS only.

Example 9

25   Maintainance of the viability of A549 cell cultures by DIPE
after periods of exposure to HD

In order to probe the efficacy of immediate post-treatment of
HD-exposed cells with DIPE, a further set of experiments was
30   carried out to determine if adding DIPE at various times after
the application of HD to the cultures would reduce the toxicity
of HD. In this work, 100 ml HD (200 mM) was added to the wells
of a 96-well plate, and at intervals of up to 20 min, 100 ml

DIPE (8 mM) in HBSS was then added to give final concentrations
of 100 mM HD and 4 mM DIPE. The effect of this treatment was
compared to cultures treated with HBSS, DIPE and HD alone. The
cultures were treated with these solutions for a total of 1 h at
5 37°C (5% CO_2/95% air), after which the solutions were removed.
The cell layer was washed with 100 ml HBSS and 100 ml of DMEM
medium added to the wells and further incubated as above.
Viability was subsequently determined at 48 h by the NR assay.
For both these experiments the data are presented as mean values
10 +/- SD (n=12). Welch's approximate two-tailed t-test (unpaired)
was used to determine if any increase in protection obtained by
treating cultures with DIPE before during or after HD exposure
was significant compared to HD only treated cultures.

15 The results are shown in Figure 9. The viability of the cultures
exposed to HD for 0.5, 2.5 and 5 min prior to treatment with
DIPE was 1.8-, 1.5- and 1.4-fold greater respectively than the
HD only treated cell cultures (p<0.0001). After 5 min, cell
viability declined, so that at 7.5 and 10 min it was 1.4- and
20 1.3-fold greater (p<0.0001) than that of unprotected cultures.
After 12.5 min, cell viability in DIPE treated cultures fell to
levels comparable to that of HD only exposed cultures.

32

Claims

1.   The use of a compound of formula (I)
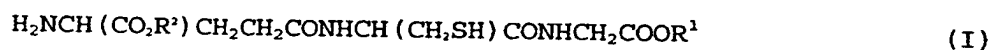
$$H_2NCH(CO_2R^2)CH_2CH_2CONHCH(CH_2SH)CONHCH_2COOR^1 \qquad (I)$$

or a salt thereof;

where R^1 and R^2 are independently selected from hydrogen or an optionally substituted hydrocarbyl group having at least three carbon atoms; provided that R^1 and R^2 are not both hydrogen, in the preparation of a composition for use in protecting against alkylating agents.

2.   The use according to claim 1 wherein R^1 and R^2 are selected from hydrogen or alkyl.

3.   The use according to claim 2 wherein said alkyl group is a C_{3-6} alkyl.

4.   The use according to any one of claims 1 to 3 wherein the compound of formula (I) is monoisopropylglutathione ester (MIPE).

5.   The use according to any one of claims 1 to 3 wherein the compound of formula (I) is diisopropylglutathione ester (DIPE).

6.   The use according to any one of the preceding claims wherein the composition is in a form which is suitable for topical application.

7.   The use according to claim 6 wherein the composition is in the form of a barrier cream.

8.   The use according to any one of claims 1 to 5 wherein the composition is in liquid form.

9.   The use according to claim 8 which is in a form suitable for administration by a nasal spray or by an inhaler.

10.   The use according to claim 9 wherein the compound of formula (I) is incorporated into vesicles or liposomes.

11.   A method of protecting living tissue from the effects of alkylating agent, said method comprising applying to the tissue, a compound of formula (I) as described above, either before, during or after exposure to said alkylating agent.

12.   A method according to claim 11 wherein said compound is applied before or during exposure to said alkylating agent.

13.   A method according to claim 12 wherein said compound is applied before and during exposure to said alkylating agent.

14.   A pharmaceutical composition comprising a compound of formula (I)
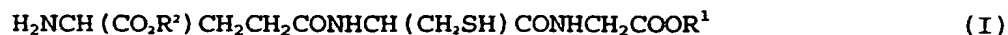
$$H_2NCH(CO_2R^2)CH_2CH_2CONHCH(CH_2SH)CONHCH_2COOR^1 \qquad (I)$$

or a salt thereof;
where R^1 and R^2 are independently selected from hydrogen or an optionally substituted hydrocarbyl group having at least three carbon atoms; provided that R^1 and R^2 are not both hydrogen, and

a pharmaceutically acceptable carrier, wherein the composition is in a form suitable for topical administration.

15.   A composition according to claim 14 which comprises a barrier cream.
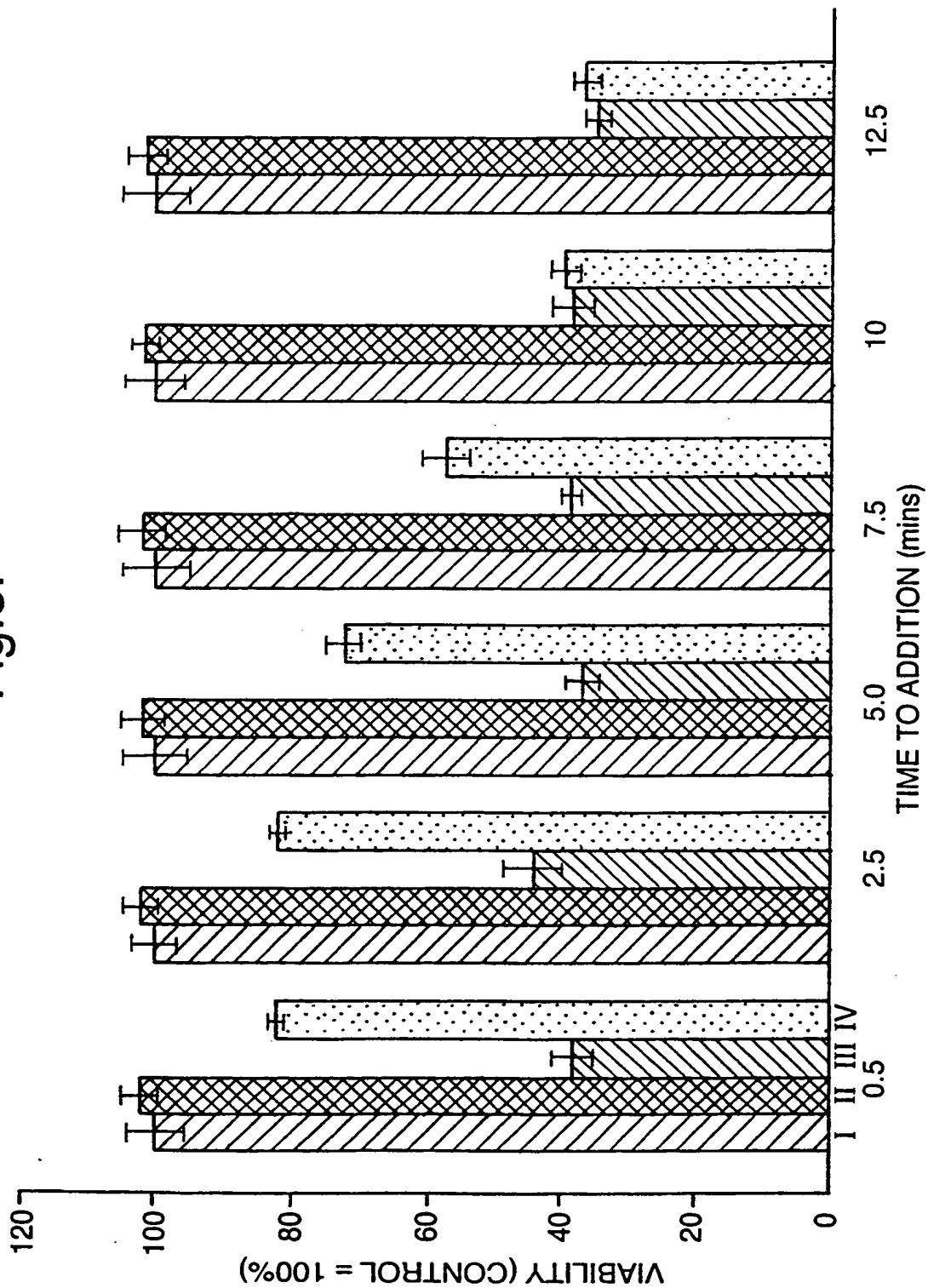
Fig.1.

Fig.2a.

(1) (2) (3)

Fig.2b.

(1) (2) (3)
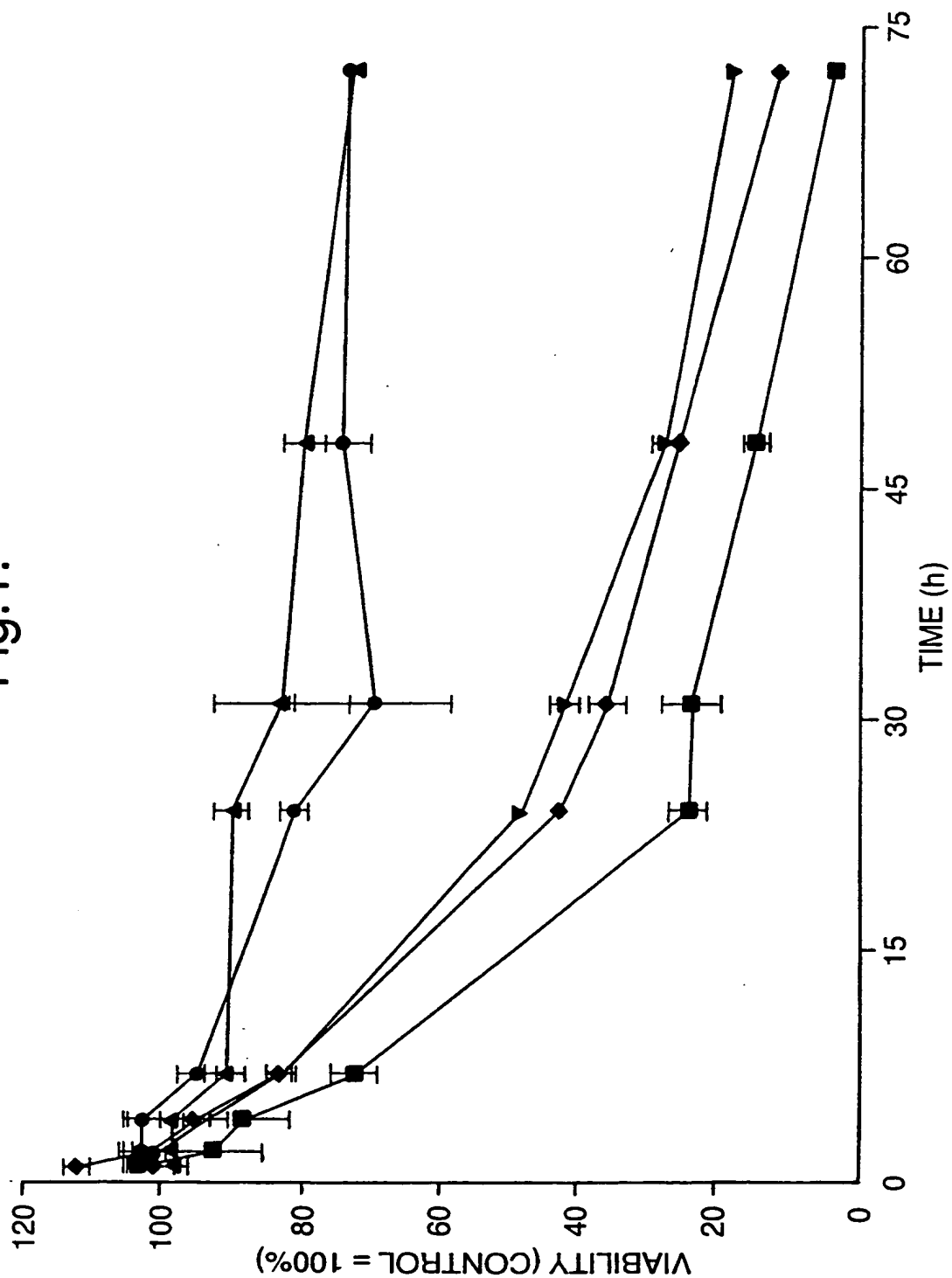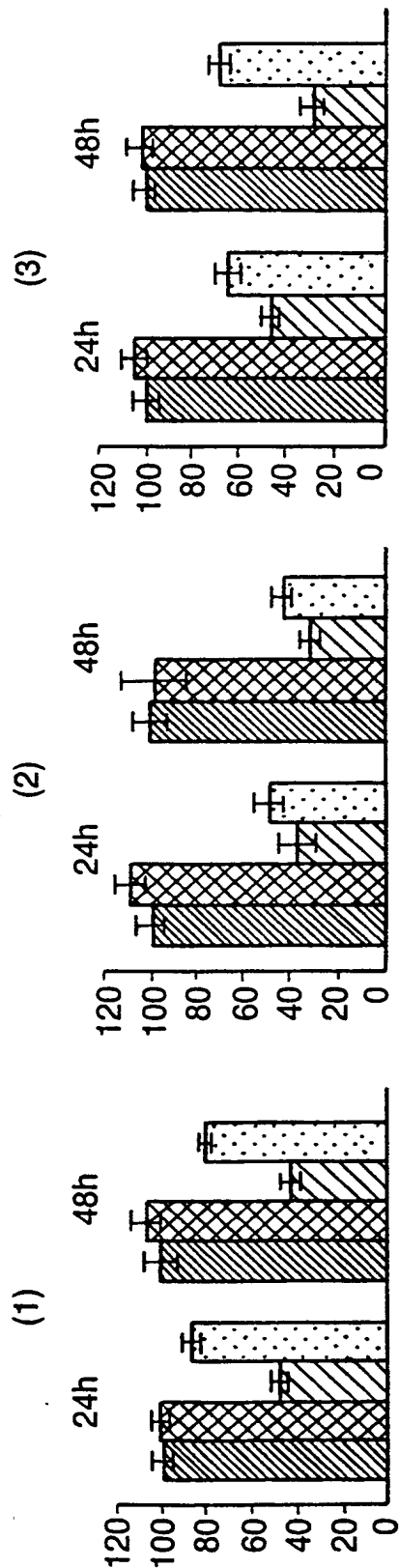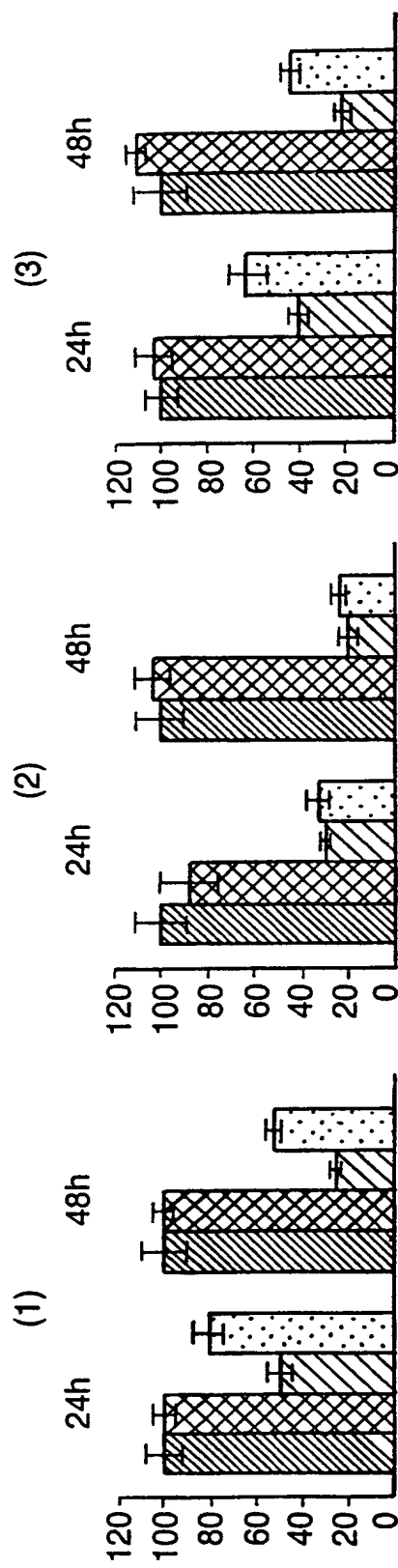
Fig.3.

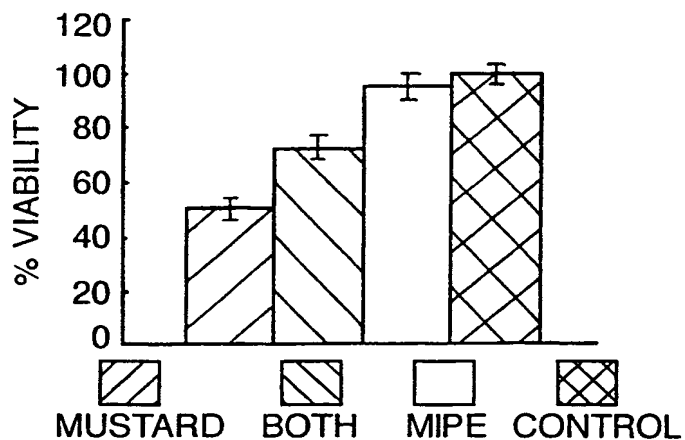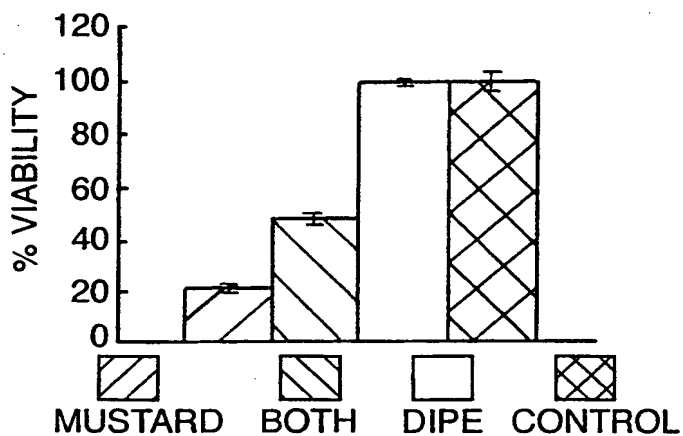Fig.4A.   Protective effects of MIPE in RPMI-2650

Fig.4B.   Protective effects of DIPE in RPMI 2650
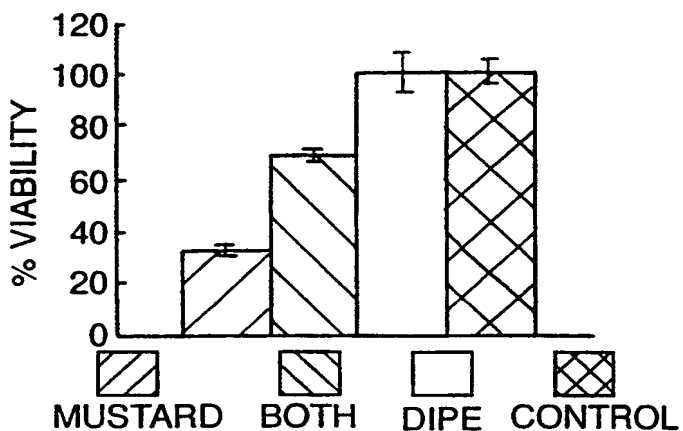

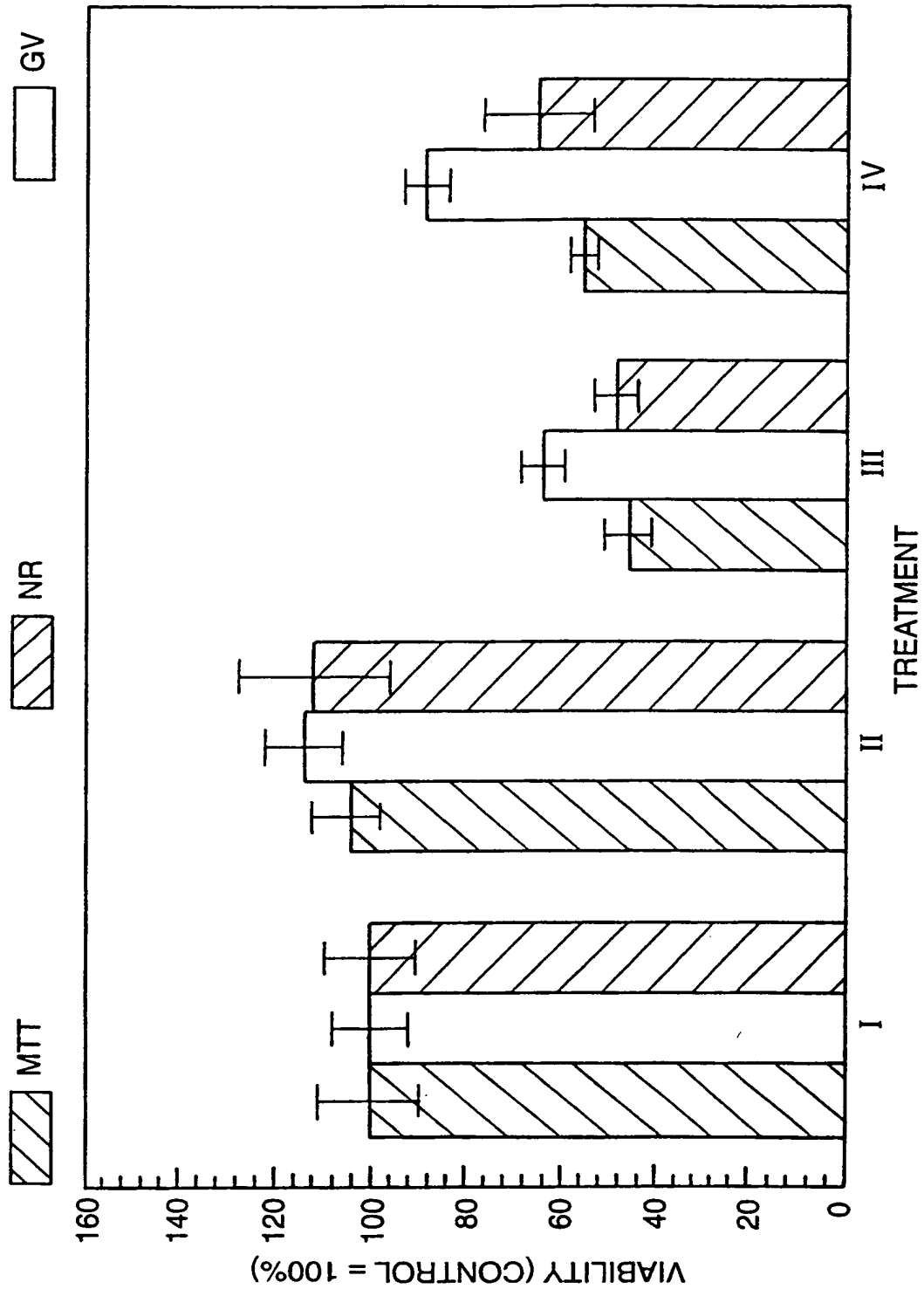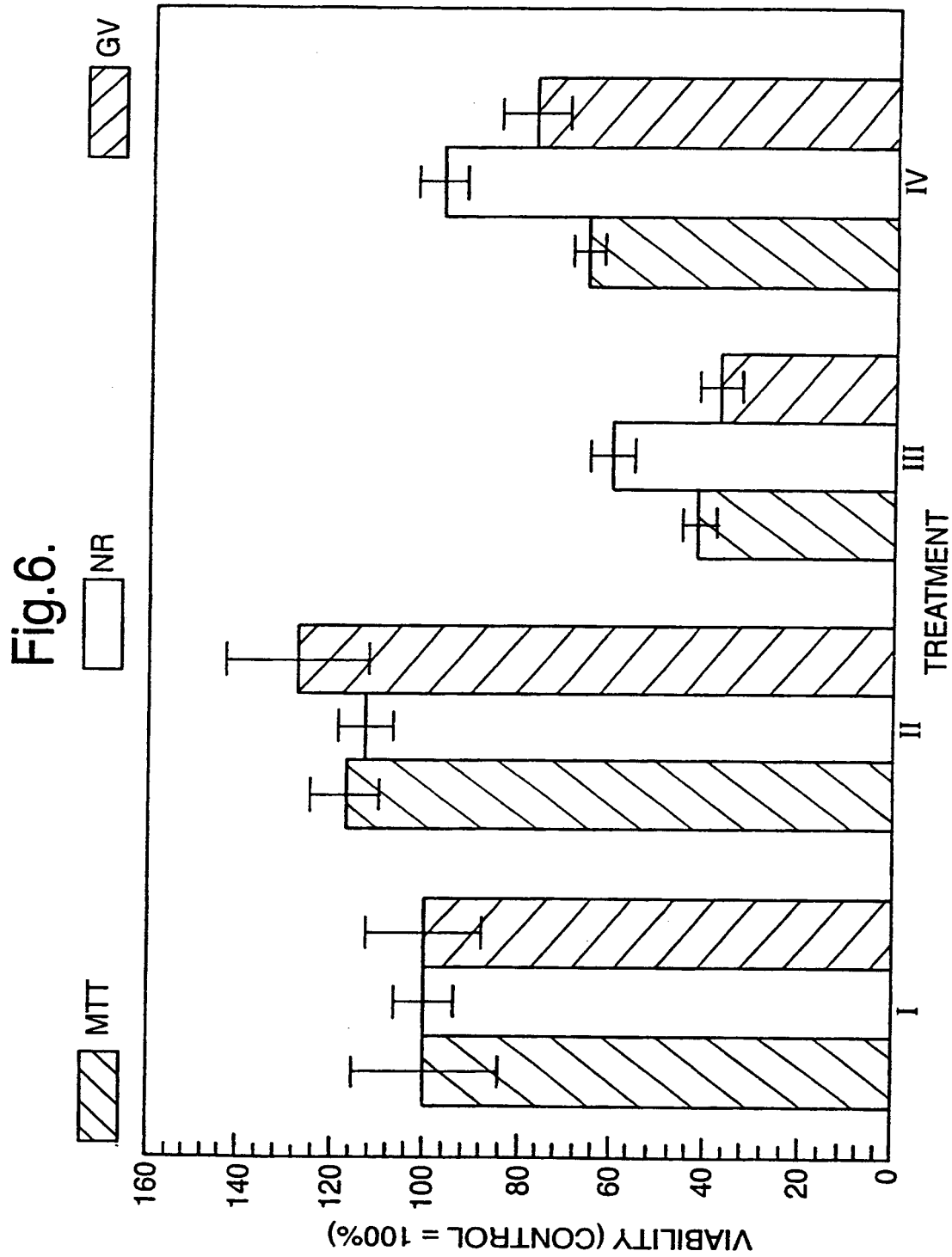Fig.4C.   Protective effects of DIPE in BEAS-2B
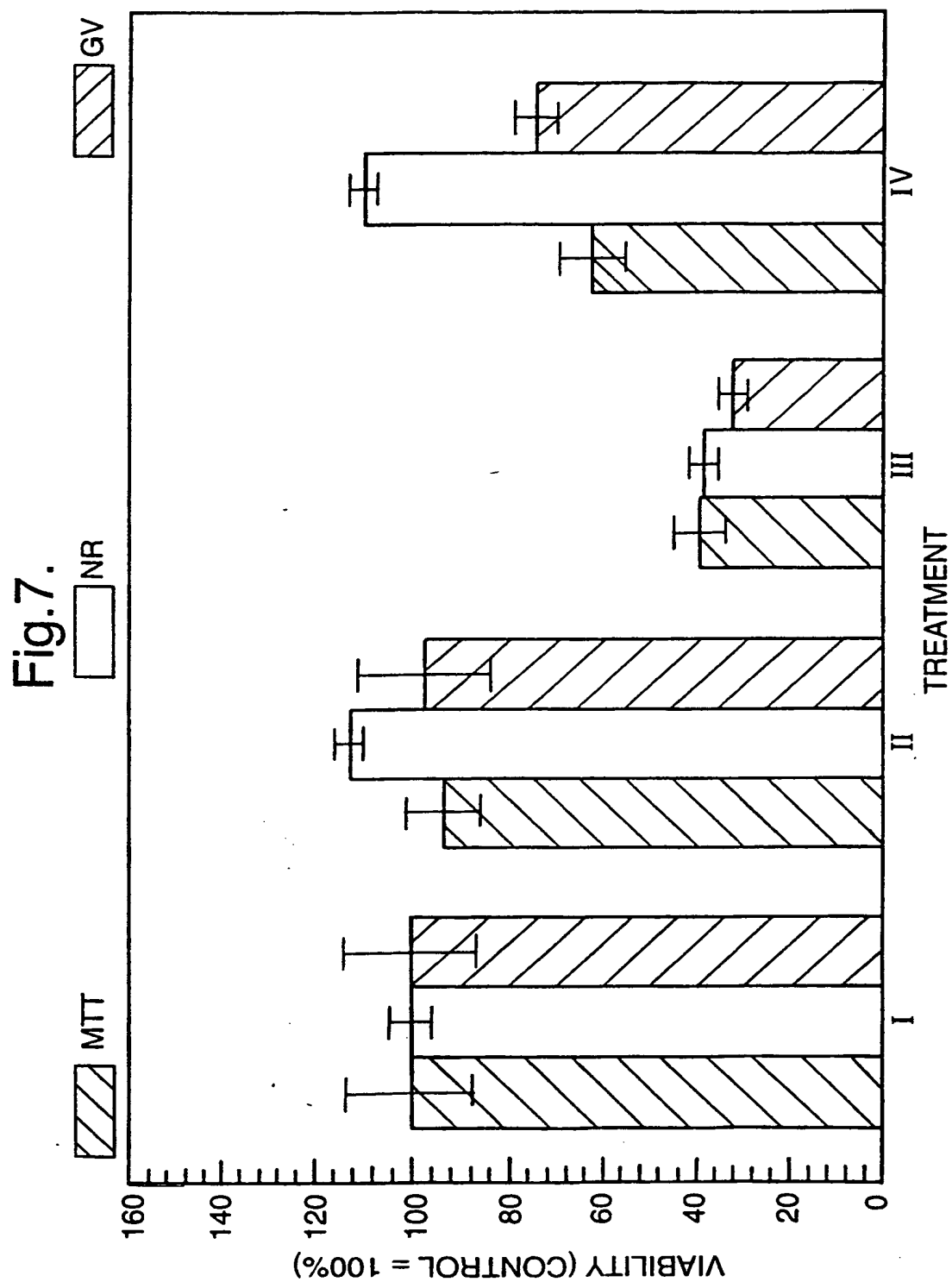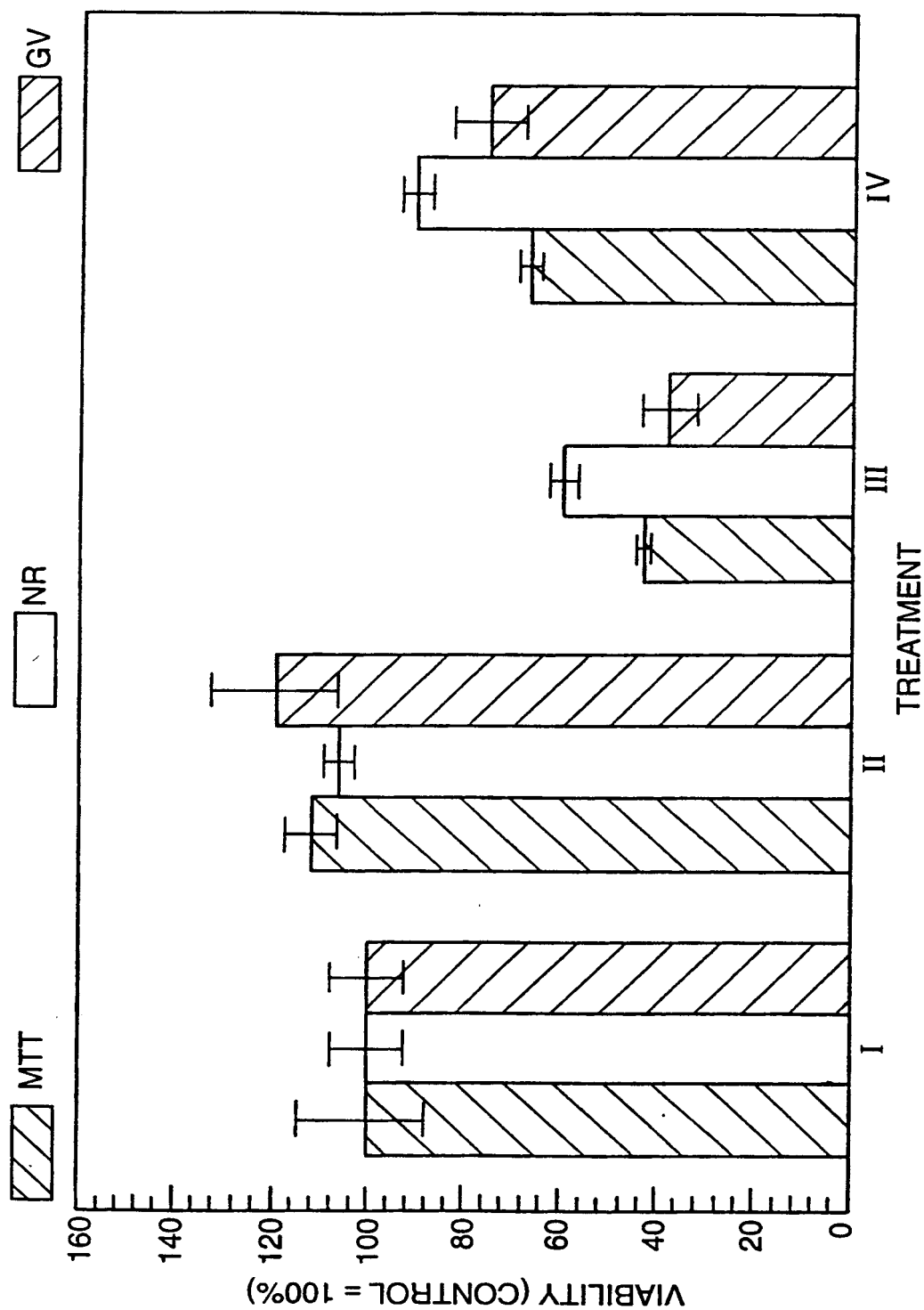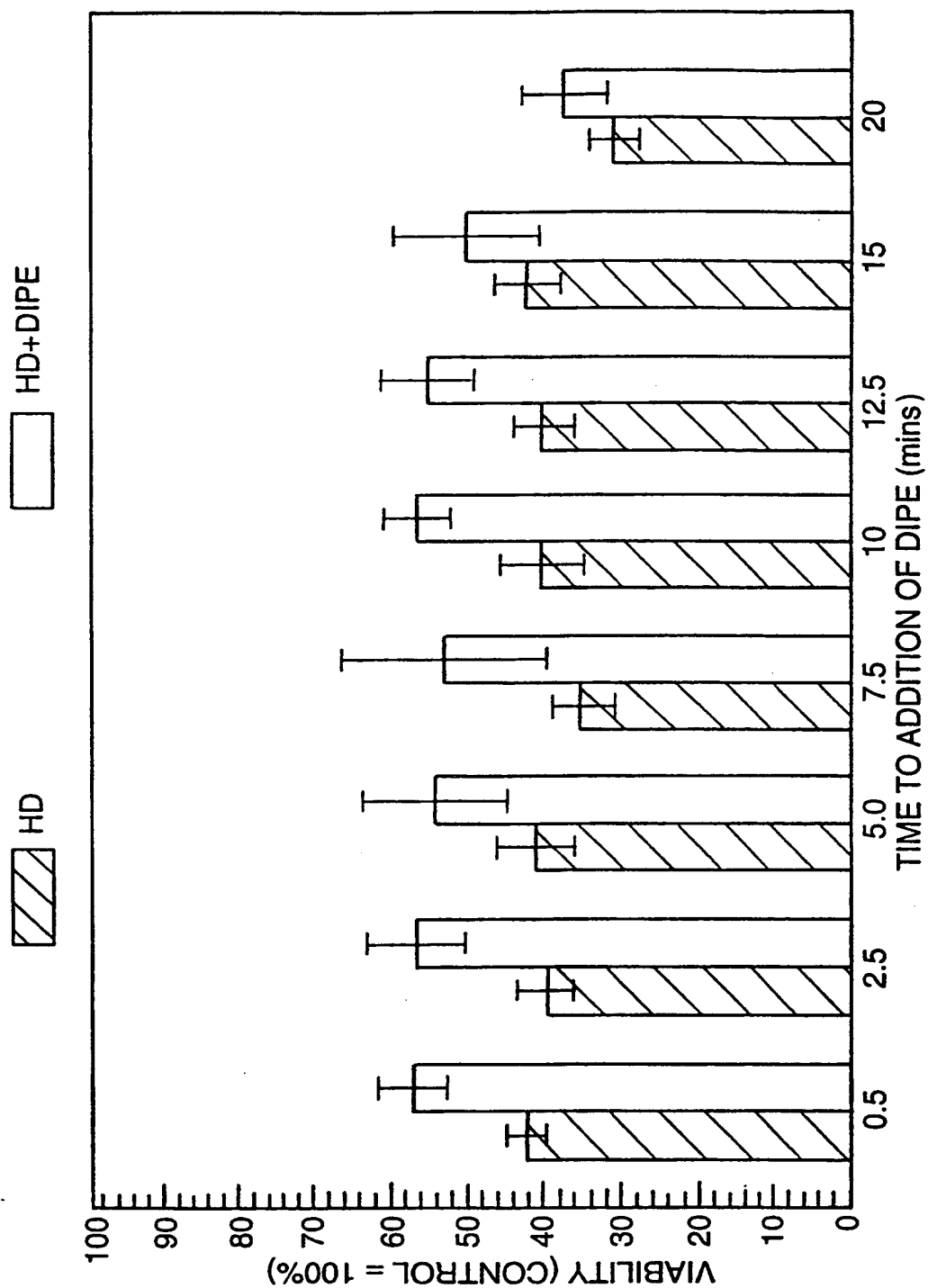
DOCID: <WO___9855135A1_I_>

Fig.5.

Legend: MTT, NR, GV

Y-axis: VIABILITY (CONTROL = 100%), 0 to 160

X-axis: TREATMENT (I, II, III, IV)

Fig.6.

DOCID: <WO___9855135A1_I_>

Fig.7.

Fig.8.

Fig.9.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61K38/06

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LINDSAY C D ET AL: "Monoisopropylglutathione ester protects A549 cells from the cytotoxic effects of sulfur mustard" HUM. EXP. TOXICOL. (HETOEA,09603271);1997; VOL.16 (11); PP.636-644, XP002079237 DERA, CBD Porton Down;Salisbury; SP4 0JQ; UK (GB)	1-4,6,8, 11-14
Y	see abstract see page 636, column 1, paragraph 1 – page 637, column 1, paragraph 2 see page 638, column 2, paragraph 2 – page 641, column 2, paragraph 1; figures 1,2,4 --- -/--	5,7,9, 10,15

| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
9 October 1998	02/11/1998

| Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx: 31 651 epo nl;
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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 04024 A (UNITED KINGDOM SECRETARY OF STATE FOR DEFENCE, LONDON;UK) 19 March 1992 see page 5, line 39 - page 7, line 2; example 2; tables 1-4	5,7,9, 10,15
X	AMIR A ET AL: "Glutathione protects J774 macrophage cells against sulfur mustard induced toxicity" MED. DEF. BIOSCI. REV., PROC. (64UTAN);1996; VOL.2,; PP.715-728, XP002079238 Israel Inst. Biological Research;Ness Ziona; Israel (IL) see abstract see page 716, paragraph 1-2; figures 1-6 see page 717, paragraph 3 - page 721, paragraph 4	1-3,6, 10-14
X	BOYD M R ET AL: "Protective role of endogenous pulmonary glutathione and other sulfhydryl compounds against lung damage by alkylating agents. Investigations with 4-ipomeanol in the rat" BIOCHEM. PHARMACOL. (BCPCA6,00062952);1982; VOL.31 (8); PP.1579-83, XP002079240 Natl. Cancer Inst.;Div. Cancer Treat.; Bethesda; 20205; MD; USA (US) see abstract	1-3
X	TAYLOR Y C ET AL: "Elevation of intracellular glutathione levels following depletion and its relationship to protection against radiation and alkylating agents" PHARMACOL. THER. (PHTHDT,01637258);1988; VOL.39 (1-3); PP.293-9, XP002079242 Stanford Univ.;Med. Cent.; Stanford; 94305; CA; USA (US) see page 297, paragraph 2 - page 298, paragraph 1; figure 6	1-3
X	ZUCALI J R ET AL: "Protective effects of human interleukin-1 on hematopoietic progenitor cells from L-phenylalanine mustard" ONCOL. REP. (OCRPEW);1995; VOL.2 (5); PP.851-6, XP002079239 Univ. Florida;Dep. Med.; Gainesville; 32610; FL; USA (US) see page 854, column 1, paragraph 2 - column 2, paragraph 1; figure 6	1-3

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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SEAGRAVE J C ET AL: "Zinc effects on glutathione metabolism relationship to zinc-induced protection from alkylating agents" BIOCHEM. PHARMACOL. (BCPCA6,00062952);1983; VOL.32 (20); PP.3017-21, XP002079241 Los Alamos Natl. Lab.;Genet. Groups; Los Alamos; 87545; NM; USA (US) see page 3020, column 2, paragraph 3 -----	1-3

INTERNATIONAL SEARCH REPORT

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:
   Remark: Although claim(s)   11-13
   is(are) directed to a method of treatment of the human/animal
   body, the search has been carried out and based on the alleged
   effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such
   an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all
   searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9204024	A	19-03-1992	AU	652183 B	18-08-1994
			AU	8525591 A	30-03-1992
			CA	2089158 A	01-03-1992
			EP	0546063 A	16-06-1993
			GB	2262446 A,B	23-06-1993
			JP	6500113 T	06-01-1994